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Incidence of *Vibrio* Species Associated with Blue Crabs (*Callinectes sapidus*) Collected from Galveston Bay, Texas

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Bacteria were readily isolated from the hemolymph of a majority (88%) of the blue crabs collected from Galveston Bay, Texas. The hemolymph of most crabs contained moderate (>10^3 bacteria/ml) to heavy (>10^5 bacteria/ml) infections. Large variances were observed in the bacterial number associated with individual crabs, but no significant difference was observed between the mean bacterial levels in the hemolymph of crabs collected during different seasons of the sampling year. *Vibrio* spp. were the predominant bacterial types in the hemolymph of infected crabs and increased in number significantly during the summer season. Warmer water temperatures were thought to be responsible for this increase. Bacterial numbers and the percentage of *Vibrio* spp. were highest in the interior of the crab bodies, especially in the digestive tract. The exterior of the crabs did not appear to be the source of the hemolymph's bacterial flora. Bacteria taxonomically identical to *Vibrio cholerae*, *V. vulnificus*, and *V. parahaemolyticus* were routinely isolated from the crab hemolymph and external carapace. *V. parahaemolyticus* was the most prevalent of the pathogenic *Vibrio* spp. and was isolated from 23% of the hemolymph samples. *V. vulnificus* (7%) and *V. cholerae* (2%) occurred less commonly in the hemolymph. The incidences of *V. parahaemolyticus* and *V. vulnificus* were related and increased in the summer months. Both organisms were frequently isolated from the same crab.

The blue crab, *Callinectes sapidus*, is commonly found in the coastal waters of the United States in the Gulf of Mexico and the Atlantic Ocean. This crab is edible and is the basis for a large seafood industry (19). Reports in the literature describing the microbial flora of the blue crab are limited, and most studies are confined to the microbial flora associated with the diseased state (15, 21, 22). In 1975, two reports describing naturally occurring bacterial flora in the hemolymph of healthy blue crabs were published (16, 20). They found that the hemolymph of a majority of the blue crabs (82%) collected from Chincoteague Bay, Virginia, contained detectable bacterial levels. Thus, unlike mammalian circulatory systems, it appeared that the circulatory system of healthy, marketable blue crabs can tolerate high numbers of bacteria in the hemolymph. Furthermore, *Vibrio* spp. were the predominant bacterial type in the hemolymph of blue crabs.

Recently, there have been reports of isolated cases of cholera in the southern United States (2, 3). Several of these outbreaks have shown a correlation between the ingestion of seafood, including crabs, and the cholera cases. In addition, a newly described marine pathogen, *Vibrio vulnificus*, has been shown to cause septicemia in humans who handle or ingest crabs (5). These reports have caused a renewed interest in the ecology of marine *Vibrio* spp. responsible for disease in humans. Since *Vibrio* spp. comprise a significant part of blue crabs' bacterial flora, an extensive survey of bacterial flora associated with blue crabs was undertaken.

The objectives of this study were: (i) to determine the level of bacteria and *Vibrio* spp. in the hemolymph of crabs collected from Galveston Bay; (ii) to examine the bacterial flora of the crab anatomy to determine the source of the hemolymph infection; and (iii) to determine the incidence of the *Vibrio* pathogens (i.e., *V. cholerae*, *V. vulnificus*, and *V. parahaemolyticus*) associated with blue crabs.

MATERIALS AND METHODS

One hundred forty crabs were collected from Galveston Bay, Texas, between November 1979 and November 1980. Crabs were collected by using trawl nets or crab traps. Once collected, crabs were returned as soon as possible to the laboratory for bacterial analysis. All crabs were alive, and most crabs were sampled within 1 h after collection.

Crab sampling. The bacterial populations of two sites on the blue crabs were routinely sampled (the ventral surface of the lateral spine and the hemolymph). A total of 81 crabs were examined. Hemo-

TABLE 1. Biochemical characteristics used to identify the pathogenic *Vibrio* spp.[a]

Test	Reaction[b]		
	V. parahaemolyticus	*V. vulnificus*	*V. cholerae*
Oxidase	+	+	+
D-Mannitol, acid	+	±	+
Inositol, acid	ND	ND	−
Sucrose, acid	−	−	+
Arabinose, acid	+	±	±
ONPG[c]	−	+	+
Salicin, acid	−	+	−
Acetylmethylcarbinol	−	−	ND
Growth 42°C	+	ND	ND
0/129 sensitivity	±	±	±
Growth in:			
0% NaCl	−	−	+
6% NaCl	+	+	±
8% NaCl	+	±	−
10% NaCl	±	±	−

[a] A few strains (3) varied by one test from these characteristics but were still classified as pathogenic *Vibrio* spp.

[b] +, Positive test; −, negative test; ±, variable reaction; ND, not determined.

[c] *o*-Nitrophenyl-β-D-galactopyranoside.

lymph samples were removed by using a sterile syringe as described by Colwell and colleagues (8).

Bacteria were isolated for identification but were not enumerated from the exterior (i.e., carapace) of the crabs. External samples were taken from the ventral surface of the lateral spine, using sterile cotton swabs premoistened with a three-salts solution.

Bacterial analyses. Total platable heterotrophic counts were determined for the hemolymph samples by the spread plate method, using a three-salts solution as a diluent and modified seawater yeast extract (MSWYE) agar. The preparation of this medium and diluent has been described elsewhere (9). The swabs used to sample the crab exterior were suspended in three-salts solution and plated onto MSWYE agar. Plates were incubated for 7 days at 25°C.

After incubation, the percentage of *Vibrio* spp. was determined for each site (e.g., hemolymph and exterior) on each crab. Between 80 and 100 colonies from each site were picked and transferred to TCBS agar (Difco Laboratories, Detroit, Mich.). The TCBS agar plates were incubated at 35°C anaerobically in GasPak chambers (BBL Microbiological Systems, Cockeysville, Md.). All isolates showing growth on TCBS agar after incubation were considered to be *Vibrio* spp., and the number of these strains relative to the number of colonies picked was used to calculate the percentage of *Vibrio* spp. in the population.

An enrichment procedure was also used to detect low levels of specific *Vibrio* pathogens associated with the crabs. A total of 140 crabs were sampled by placing a portion (0.1 to 0.5 ml) of the original hemolymph sample (or a swab of the external carapace) into alkaline peptone broth. The stepwise procedure for the enrichment of *Vibrio* spp. described by Colwell and Kaper (7) was then used. To reduce the number of isolates, individual colonies were picked and inoculated onto a screening medium (12) used to identify presumptive *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus*. Any isolate which was presumptively identified as one of the three *Vibrio* species was saved for further taxonomic analysis.

Identification of pathogenic *Vibrio* spp. Isolates identified as presumptive pathogenic *Vibrio* spp. on the screening media were subjected to a battery of biochemical tests. The techniques used for the tests have been described elsewhere (9, 16).

All isolates were gram negative, had polar monotrichous flagella, were catalase positive, produced acid but not gas from glucose, were L-lysine and L-ornithine decarboxylase positive but L-arginine dihydrolase negative, did not produce H_2S, grew at 35°C, and were urease negative but indole positive. Table 1 lists the additional tests used to classify a strain as one of the three *Vibrio* spp. pathogens.

Distribution of bacteria associated with the blue crab anatomy. The bacterial flora of 8 anatomical areas, 4 within the body cavity and 4 on the exterior surface, of 10 freshly collected crabs was examined.

The external sample sites on the crab included the carapace below the abdomen, the eye stalk, the mouth, and the ventral surface of the lateral spine. The percentages of *Vibrio* spp. on the external sites were determined by the procedure described above.

The four internal sampling sites were the heart, hemolymph, gills, and stomach. By using aseptic technique, the dorsal carapace of the crab was removed to expose the internal organs. Hemolymph (1 ml) for bacteriological examination was removed from the pericardial sinus with a syringe. The gills, heart, and stomach were aseptically removed and homogenated in diluent before enumeration.

Total platable counts and *Vibrio* spp. counts were determined for the four internal samples by the techniques described above. Between 700 to 800 bacterial isolates were examined from each crab.

All statistical differences were determined by the RXC tests of independence (18), except when comparing the relationship between *Vibrio* spp. on the interior and exterior of the crab. In this instance, the Mann-Whitney *U*-test was used to determine significant differences.

RESULTS

The hemolymph of blue crabs collected from Galveston Bay commonly contained bacterial infections. During this 12-month study, the bacterial levels of the hemolymph of 81 crabs were determined. The level of infection was found to range from a sterile hemolymph to concentrations greater than 3×10^7 bacteria/ml of hemolymph (Table 2), with a yearly geometric mean of 2.4×10^4 bacteria/ml.

For the purpose of comparison, crabs were classified into four arbitrary categories based on the level of infections in their hemolymph: sterile, lightly infected ($<10^3$ bacteria/ml), moderately infected (10^3 to 10^5 bacteria/ml), and heavily infected ($>10^5$ bacteria/ml). Approximately 77% of the crabs contained moderate (52%) or

TABLE 2. Seasonal variation in the number of bacteria associated with the blue crab hemolymph

Season	No. of crabs	Bacterial incidence in the hemolymph		
		Geometric mean ($\times 10^4$)	Arithmetic mean	Range in infected crabs
Winter	15	3.1	4.4×10^5	6.3×10^1–2.0×10^6
Spring	19	1.2	1.1×10^5	1.3×10^2–5.4×10^5
Summer	29	3.7	1.2×10^6	8.6×10^1–3.0×10^7
Fall	18	8.3	6.6×10^5	6.0×10^3–8.3×10^6
Annual	81	2.4	6.7×10^5	6.3×10^1–3.0×10^7

heavy (25%) infections in their hemolymph, whereas only 22% of the hemolymphs were sterile (12%) or lightly infected (10%).

Seasonal differences in hemolymph infections were also examined. Although mean (geometric) bacterial levels in the crab hemolymph were slightly higher in the summer and fall (Table 2), no significant differences ($P < 0.05$) were noted in these mean levels between different seasons. Bacterial infections varied over a wide range during a single season, and this large variance made detection of seasonal differences difficult.

In this study, no statistical differences were detected in the bacterial numbers in the hemolymph of injured compared with uninjured crabs, but the hemolymphs of injured crabs were rarely sterile (2% compared with an overall average of 12%). Also, the hemolymph was found to be significantly more heavily infected ($>10^5$ bacteria/ml of hemolymph) in male than in female crabs ($P < 0.05$).

***Vibrio* spp. associated with blue crabs.** *Vibrio* spp. were the predominant platable bacterial types in the crab hemolymph (Table 3). Mixed populations of bacteria, including *Vibrio* spp., were routinely isolated from the crab hemolymph. Pure cultures of *Vibrio* spp. were isolated from two crabs with heavy bacterial infections ($>10^6$). The predominant bacterial type (68% of the *Vibrio* spp. isolates) in the blue crab hemolymph produced green colonies on TCBS (i.e., could not ferment sucrose).

Changes in the season did not significantly alter total bacterial levels in the hemolymph, but the percentage of *Vibrio* spp. present did change. Mean *Vibrio* spp. levels in the crab hemolymph remained constant (approximately 57%) during the winter, spring, and fall (Table 3). During the summer, the average *Vibrio* spp. levels (70%) increased and were significantly higher ($P < 0.05$) than during the other three seasons.

TABLE 3. Seasonal variation in the percentage of *Vibrio* spp. associated with blue crabs

Source	% *Vibrio* spp. (mean ± SD)			
	Winter	Spring	Summer	Fall
Hemolymph	52 ± 29	60 ± 28	70 ± 24	57 ± 38
Exterior	33 ± 23	19 ± 11	23 ± 29	22 ± 25

Unlike the hemolymph, *Vibrio* spp. comprised a minor percentage of the total bacterial flora of the crab exoskeleton (Table 3). *Vibrio* spp. routinely comprised 25% of the total bacterial flora associated with the exoskeleton, and seasonal differences were small. During the summer and fall, in approximately 45% of the crabs sampled, *Vibrio* spp. comprised less than 10% of the bacterial populations associated with the exterior. During the winter months, a slight increase was observed. Furthermore, a comparison of the *Vibrio* spp. located internally versus externally demonstrated a significantly higher ($P < 0.05$) population in the hemolymph during the spring, summer, and fall. During the winter, no differences were found.

Characterization of the bacterial flora of the crab anatomy. The percentage of *Vibrio* spp. was found to be approximately the same (5.5 ± 5%) on four different sites on the exterior of the 10 crabs sampled.

The internal bacterial flora of the crab varied with the area of the anatomy sampled. The mean value for hemolymph infections (7.2×10^4 bacteria/ml) for these 10 crabs was similar to the yearly mean (2.4×10^4 bacteria/ml). The heart was similar to the hemolymph in its level of bacterial infections (10^5 bacteria/g) and the percentage of *Vibrio* spp. (21 and 18%, respectively). The bacterial flora of the crab stomach and gills was different than in the heart and hemolymph. Both the stomach and gills were heavily infected with bacteria (4.7×10^8 and 7.9×10^8 bacteria/g, respectively). Although the total *Vibrio* spp. levels were approximately the same for the gills (2.2×10^8 *Vibrio*/g) and for the stomach (3.5×10^8 *Vibrio*/g), *Vibrio* spp. comprised 75% of the stomach flora, but only 30% of the gill flora.

***Vibrio* pathogens.** The hemolymph and exterior carapace of 140 blue crabs were examined through an enrichment technique for the inci-

TABLE 4. Seasonal incidence of pathogenic *Vibrio* spp. associated with blue crabs

Source	Pathogen	% of crabs infected/season				
		Winter	Spring	Summer	Fall	Annual[a]
Hemolymph	*V. parahaemolyticus*	7	0	40	24	23
	V. vulnificus	7	0	11	4	7
	V. cholerae	11	0	0	0	2
Exterior	*V. parahaemolyticus*	11	0	9	9	8
	V. vulnificus	4	0	4	0	2
	V. cholerae	0	11	0	0	1.5

[a] Calculated from a total crab sample of 140.

dence of specific *Vibrio* pathogens. Bacteria taxonomically resembling *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus* were routinely isolated from the hemolymph and external carapace (Table 4). These pathogens were isolated from 38% of the total crabs sampled: 27% of the hemolymph samples and 11% of the exoskeleton samples. Only once was a potential pathogen (*V. parahaemolyticus*) isolated from external and internal sites on the same crab. *V. cholerae* and *V. vulnificus* were rarely isolated from crab samples (3.5 and 9%, respectively), whereas *V. parahaemolyticus* was isolated from 30% of the crab samples.

Bacteria biochemically resembling *V. cholerae* were isolated from five crabs that were collected in the winter and early spring. Two of the five isolates were also identified as *V. cholerae* with API 20E biochemical strips. After biochemical identification, serological identification of the five *V. cholerae* isolates was attempted. None of the isolates were typed as O-1 *V. cholerae*.

V. cholerae and *V. parahaemolyticus* were never isolated from the same crab sample. Conversely, *V. vulnificus* and *V. parahaemolyticus* were routinely isolated from the same crabs. Fifty percent of the *V. vulnificus* isolates came from crabs containing *V. parahaemolyticus*. Furthermore, significantly greater numbers of *V. vulnificus* and *V. parahaemolyticus* were isolated during the summer and fall ($P < 0.01$) (Table 4). In general, *Vibrio* pathogens were associated with over 50% of the blue crabs sampled during the summer months and 35% of the crabs sampled in the fall. Additionally, there was a significant association between these pathogens and injured crabs ($0.1 > P > 0.05$).

DISCUSSION

In this study, the bacterial burdens of most crabs were high but generally in the range reported in the literature (16, 20). No significant difference was noted in bacterial levels in the hemolymph of crabs collected during different seasons. Other workers reported a decrease in the bacterial levels in the hemolymph of crabs collected from Chesapeake Bay when the water temperature fell below 15 to 20°C (16). In this study, no significant difference between the bacterial levels of crabs collected from water at temperatures above and below 20°C was noted, possibly because of the warmer winter water temperatures of Galveston Bay.

Most (greater than 75%) of the crabs collected from Galveston Bay were moderately or heavily infected, whereas only a few (12%) were bacteria-free. Whether bacterial infections of the hemolymph are a short-term phenomenon or a stable trait is unknown. One hypothesis is that the immune system of crabs is slow acting. Any bacteria which penetrate into the hemolymph would proliferate in the hemolymph for a brief period before being removed by the crabs' immune mechanisms. Bacteria which grew rapidly (e.g., *Vibrio* spp. in the warmer months) would have a natural advantage.

Heavy bacterial burdens were found more often in the hemolymph of male than of female crabs. The reason for this increased bacterial burden is not understood, but was not due to an increased incidence of injury among male crabs as was reported in Chesapeake Bay crabs (16).

There were large variations in the percentage of *Vibrio* spp. (0 to 100%) present in different crabs. However, *Vibrio* spp. were the predominant bacterial type in the hemolymph of most crabs. In approximately 40% of the crabs sampled, *Vibrio* spp. made up at least 75% of the bacterial numbers in the hemolymph. During the summer season, there was a significant increase in the percentage and actual number of colony-forming units classified as *Vibrio* spp. Since the mean bacterial levels did not increase in the crab hemolymph during the summer, a concomitant decrease in other bacterial types must have occurred. Increased *Vibrio* spp. levels were probably due to the elevated water temperatures, as has been reported elsewhere (10).

In a previous study, the *Vibrio* spp. found in crab hemolymphs were assumed to have originated on the exterior of the crabs and entered the crab hemolymph through injuries (16). The external and internal bacterial flora of the crabs

examined in this study were significantly different, at least in terms of percentage of *Vibrio* spp. Thus, it appears that either blue crab hemolymph selects for *Vibrio* spp. or another part of the crab anatomy is perhaps the source of the hemolymph's bacterial flora. The open circulatory system of blue crabs causes problems in determining the origin of any bacteria in the hemolymph. However, in the 10 crabs examined in detail, the percentage of *Vibrio* spp. composition of the hemolymph closely resembled the microflora of the crabs' digestive tract. We postulate that the bacteria in blue crab hemolymph originates in the crabs' digestive system and enters the hemolymph perhaps when migrating parasites perforate the gut wall. Other investigators (13, 14, 17) have proposed that *Vibrio* spp. are the major bacterial type in the gut of marine animals. This study supports the hypothesis that *Vibrio* spp. selectively colonize the stomach of the crab and may be considered as the predominant enteric organisms in blue crabs.

Recent cholera outbreaks in Louisiana and Texas have been associated with the ingestion of seafood, including blue crabs (4). These outbreaks have led to a renewed interest in the association of pathogenic *Vibrio* spp. associated with edible marine organisms. Bacteria taxonomically resembling *V. cholerae*, *V. vulnificus*, and *V. parahaemolyticus* are routinely associated with the type of blue crab sampled in this study. *V. parahaemolyticus* and *V. vulnificus* were isolated more often from the hemolymph than from the exterior carapace. The crab hemolymph appears to constitute a suitable environment for the proliferation of these pathogenic *Vibrio* spp.

V. cholerae, *V. parahaemolyticus*, and *V. vulnificus* were isolated at different frequencies. *V. cholerae* was isolated from 3.5% of the crabs sampled and only during the cooler months (spring and winter). Kaper and colleagues (11) reported similar results for the incidence of *V. cholerae* in the Chesapeake Bay.

Some of the isolates collected during this study were biochemically identical to *V. cholerae* (as described by Table 1 and the API profile index), but serological identification of these isolates was inconclusive. The *V. cholerae* isolates failed to agglutinate in any of the available *V. cholerae* antisera (O-1 or non-O-1 antisera). Nonagglutinable *V. cholerae*-like microorganisms similar to those isolated in this study have been reported to be pathogenic to humans (1).

Unlike *V. cholerae*, *V. vulnificus* and *V. parahaemolyticus* were isolated from crabs primarily during the warmer seasons (summer and fall). Indeed, *V. parahaemolyticus* and *V. vulnificus* were isolated from over 50% of the crabs collected from Galveston Bay during the summer when warm water temperatures were optimal for the growth of these organisms.

The incidences of *V. parahaemolyticus* and *V. vulnificus* were closely related. Over 50% of the crabs with *V. vulnificus* in their hemolymph also contained *V. parahaemolyticus*. The occurrence of two different pathogenic *Vibrio* spp. simultaneously in the crab hemolymph is important both ecologically and from a public health viewpoint. *V. parahaemolyticus* and *V. vulnificus* are taxonomically very similar and appear to occupy the same ecological niche.

Oral doses of 10^4 to 10^8 *V. cholerae* organisms can routinely induce cholera infections in humans (6). In this study, the levels of pathogenic *Vibrio* spp. in crabs were not determined directly. However, in many crabs, *Vibrio* spp. comprised the majority of the total bacterial flora.

Pure cultures of *Vibrio* spp. at the level of greater than 10^6 *Vibrio* spp./ml were isolated from the hemolymph of two different crabs. Although these isolates were not pathogens, *V. cholerae* (or other *Vibrio* pathogens) may reach similar levels in the crab hemolymph, and thus the hemolymph could easily contain infective doses. Also, the *Vibrio* spp. levels in the stomach and gills routinely reach concentrations greater than 10^8 *Vibrio* spp./g. Once again, these levels are sufficient to constitute an infective dose if even 1% of the *Vibrio* spp. are pathogens.

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